

# Polymer brushes and self-assembled monolayers: Versatile platforms to control cell adhesion to biomaterials (Review)

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This review focuses on the surface modification of substrates with self-assembled monolayers (SAMs) and polymer brushes to tailor interactions with biological systems and to thereby enhance their performance in bioapplications. Surface modification of biomedical implants promotes improved biocompatibility and enhanced implant integration with the host. While SAMs of alkanethiols on gold substrates successfully prevent nonspecific protein adsorption *in vitro* and can further be modified to tether ligands to control *in vitro* cell adhesion, extracellular matrix assembly, and cellular differentiation, this model system suffers from lack of stability *in vivo*. To overcome this limitation, highly tuned polymer brushes have been used as more robust coatings on a greater variety of biologically relevant substrates, including titanium, the current orthopedic clinical standard. In order to improve implant-bone integration, the authors modified titanium implants with a robust SAM on which surface-initiated atom transfer radical polymerization was performed, yielding oligo(ethylene glycol) methacrylate brushes. These brushes afforded the ability to tether bioactive ligands, which effectively promoted bone cell differentiation *in vitro* and supported significantly better *in vivo* functional implant integration. © 2009 American Vacuum Society. [DOI: 10.1116/1.3089252]

## I. INTRODUCTION: MODIFICATION OF SYNTHETIC SURFACES TO MIMIC BIOLOGICAL FUNCTIONS

Silicone, titanium, Teflon, and stainless steel have found widespread use in medical implants. However, these materials elicit inflammatory responses, including a foreign body response and fibrous encapsulation, which lead to suboptimal integration and biological performance of the implanted device.<sup>1–5</sup> Following implantation, synthetic materials undergo dynamic adsorption of proteins and other biomolecules which induce inflammatory cell responses.<sup>6</sup> While coatings and other treatments have been developed to address these limitations, many materials still provide little control over the adsorption of proteins and other biomacromolecules that occurs upon contact with biological fluids. This work led to a new paradigm in biomaterials research which focuses on methods to control the presentation of biomacromolecules and cells onto the surfaces of materials. This is achieved, in part, by the development of biomimetic materials which present bioligands within a protein-adsorption resistant non-fouling background.<sup>7–10</sup>

Adhesion of cells to a substrate is a complex process that involves protein adsorption to a surface and presentation of

specific peptide sequences (“adhesion sequences”). Upon implantation of medical devices, proteins such as fibrinogen and immunoglobulins are nonspecifically adsorbed from physiological fluids onto the material surface, Fig. 1.<sup>11–13</sup> Receptors on cell surfaces, called integrins, are transmembrane proteins that adhere to specific peptide sequences presented by the adsorbed proteins. Binding to the integrin triggers a number of cellular responses which subsequently control inflammation, tissue formation, and incorporation of the implant into the host.<sup>14–16</sup> Surfaces can experience rapid, non-specific, and reversible adsorption of proteins which may give rise to uncontrolled cell adhesion. Factors that influence cell adhesion to substrates include the density of adsorbed protein and the spatial relationship between synergistic adhesion sequences.<sup>17–22</sup> In addition, the composition and density of the biomolecules on the surface may change dynamically due to competitive adsorption and rearrangement (the “Vroman effect”) and by cell-mediated protein deposition and reorganization. Conformational changes may lead to denaturing and loss of biological activity of the protein.<sup>23</sup> Thus, it is highly desirable to attain control over the manner in which proteins adsorb onto substrates through the molecular design of interfaces.

Attempts to control cellular responses by the development of biomimetic materials have focused on presentation of bioactive peptide sequences. These sequences mimic the functions of biological molecules found in the extracellular matrix (ECM). However, these approaches often do not address the issue of nonspecific protein adsorption, the density of

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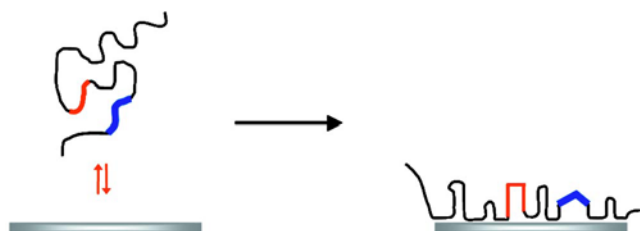


FIG. 1. (Color online) Proteins undergo conformational changes upon adsorption to a synthetic substrate, thereby exposing adhesion sequences which mediate cell adhesion.

adhesive peptides on the surface, or the importance of dynamic nature that is essential to formation and function of the ECM.<sup>7–10</sup>

The ECM consists of a complex network of proteins and polysaccharides that are secreted and arranged by cells.<sup>24</sup> Since the ECM is a key structural and functional component of tissues, it continually undergoes changes to ensure maintenance of its structure and presentation of growth factors and adhesion sequences such as those in collagen (COL) and fibronectin (FN). Structural proteins such as COL and elastin form a matrix which provides structural support for cells. Other components provide cues for signaling cell regulation, migration, and proliferation,<sup>24–31</sup> thereby influencing tissue development, blood clotting, wound healing, and cancer metastasis.<sup>32–35</sup> These features motivate research whereby new biomaterials are designed that present specific peptide sequences as a mimic of the ECM.

One of the most widely studied and well-characterized ECM proteins is FN.<sup>36–39</sup> The plasma form of FN is a glycoprotein consisting of two 220 kDa subunits that are connected by disulfide bonds. FN is soluble in blood plasma and is assembled into the ECM to form insoluble fibrils by creation of a multimer.<sup>40</sup> This process requires that cells be adhered to the ECM and integrin receptors participate in the organization of FN into the fibrils.<sup>41</sup> FN fibrils in the ECM regulate many cell functions such as gene expression, cell cycle progression, and differentiation. FN fibrils are responsible for assembling other proteins in the ECM, and they play central roles in embryonic development, tissue formation, homeostasis, and repair.<sup>42,43</sup>

Proteins in the ECM contain sequences which promote cell adhesion. These include arginine-glycine-aspartic acid<sup>44</sup> (RGD) in FN and glycine-phenylalanine-hydroxyproline-glycine-glutamate-arginine<sup>45,46</sup> (GFOGER) in COL. RGD is a ubiquitous cell binding sequence. Many integrin receptors recognize this sequence, thereby facilitating adhesion of many cell types to FN in the ECM.<sup>44</sup> COL is abundant in mesenchymal tissues and the GFOGER sequence promotes cell adhesion and osteoblast differentiation.<sup>47</sup> An understanding of the ability of these adhesive sequences to exert control over cell adhesion provides an opportunity to design biomimetic materials for medical implants and thereby promote better incorporation of the device into the host.

Modification of the surfaces of materials used in medical applications by adsorption or covalent tethering of adhesive peptides such as RGD and GFOGER promotes cell adhesion and migration.<sup>48–51</sup> However, further consideration must also be given to the density and spatial arrangement of adhesion peptides.<sup>52–56</sup> In FN, the RGD adhesion sequence is located in close proximity to a synergistic proline-histidine-serine-arginine-asparagine (PHSRN) sequence. This synergistic binding site enhances integrin receptor binding specificity and affinity and aids in the promotion of cell adhesion, spreading, and differentiation.<sup>57–63</sup> Thus, modification of surfaces with short peptide sequences that contain RGD but do not provide the synergistic site suffers from decreased biological activity.<sup>64–66</sup>

While a great deal of success has been achieved in mimicking some of the functions of the ECM, it is clear that the development of biomaterials which possess the complex functionality of the ECM remains elusive. For example, little research has focused on the design of materials that promote the complex processes involved in the cell-mediated assembly of protein matrices. Thus, we have developed a number of methods to impart metallic surfaces (gold in model systems and titanium in studies directed toward the development of new bone implants) with resistance to nonspecific adsorption of proteins and by subsequently functionalizing the surface by covalently attaching an adhesive peptide sequence, such as RGD or GFOGER, cell adhesion and differentiation can be directed to elicit specific responses to enhance integration of the implant into the host.

## II. SELF-ASSEMBLED MONOLAYERS AS MODELS FOR BIOLOGICAL INTERFACES

### A. Self-assembled monolayers

A self-assembled monolayer (SAM) is formed when molecules in solution or the vapor phase adsorb and spontaneously organize into a single layer on a surface. SAMs are formed by adsorption of a variety of functional organic molecules onto suitable solid substrates. Alkanethiols assemble on gold,<sup>67–70</sup> silver,<sup>71,72</sup> copper,<sup>73</sup> palladium,<sup>74</sup> and platinum<sup>75,76</sup> to provide densely packed molecular monolayers, Fig. 2(A). Other combinations of adsorbates and substrates include chlorosilanes on silicon oxide,<sup>77,78</sup> aluminum,<sup>79</sup> and titanium,<sup>80</sup> phosphonic acids on aluminum<sup>79</sup> or titanium,<sup>81,82</sup> and catechol derivatives on titanium.<sup>83</sup> Although alkanethiol SAMs on gold are most commonly studied, they suffer from instability of the thiol-gold bond leading to facile exchange of the adsorbates. Adsorption of trichlorosilanes on a variety of oxide surfaces is irreversible, but traces of water lead to the deposition of ill-defined multilayers through formation of siloxane linkages (Si–O–Si).<sup>84,85</sup> Use of a monochlorosilane avoids this complication, Fig. 2(B).

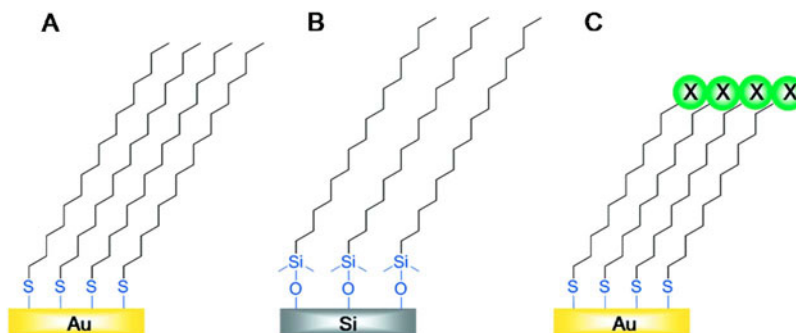


FIG. 2. (Color online) Self-assembled monolayers. (A) Alkanethiols on gold substrate. (B) Alkyl silane monolayer formed by treatment of oxidized silicon surface with alkylchlorodimethylsilane. (C)  $\omega$ -functionalized alkanethiol on gold.

## B. Controlled adsorption of proteins on self-assembled monolayers

SAMs of alkanethiols on gold provide suitable systems to explore the effects of surface chemistry on protein adsorption,<sup>86–89</sup> albeit these are largely restricted to *in vitro* analyses owing to the long-term instability of these assemblies. Long-chain alkanethiols bearing a terminal functional group [i.e.,  $\text{HS}-(\text{CH}_2)_n-\text{X}$ , where  $n \geq 10$ ] spontaneously assemble onto gold to form densely packed and ordered monolayers, Fig. 2(C).<sup>86,90–92</sup> The physicochemical properties of the monolayers are determined by the identity of the terminal functionality of the adsorbate,<sup>93,94</sup> as demonstrated, for example, by its effect on wetting.<sup>95–98</sup> Recently, SAMs have also been used as model systems for the design of biosensors<sup>67,99–102</sup> and nanoscale switchable surfaces.<sup>103,104</sup> The simplicity of creating surfaces presenting a wide range of chemistries makes the use of SAMs an attractive approach to study interfacial interactions for numerous applications.

The deposition of SAMs has been studied extensively as a method to control interactions between solid substrates and biological systems, including the influence of surface chemistry on protein adsorption<sup>105–110</sup> and cell adhesion.<sup>111–115</sup> For example, SAMs of thiols bearing terminal carbohydrates [e.g., Fig. 2(C),  $\text{X}=\text{agarose}$  or  $\text{mannitol}$ ] prevent protein adsorption and cell adhesion to gold substrates for up to 25 days *in vitro*.<sup>69,105</sup>

Alkanethiol SAMs with an oligo(ethylene glycol) (OEG) chain at the termini prevent protein adsorption and cell adhesion.<sup>67–69,71,107,116</sup> For example, monolayers of  $\text{HS}-(\text{CH}_2)_{11}-(\text{OCH}_2\text{CH}_2)_n-\text{OH}$  (abbreviated  $\text{EG}_n$ ), where  $n=3,6$ , have been studied extensively, Fig. 3. The amount of protein adsorption to gold substrates modified with mixed SAMs consisting of  $\text{EG}_n$  and an unfunctionalized alkanethiol coadsorbate is a function of the density of  $\text{EG}_n$  adsorbates and the length of the terminal OEG oligomers.<sup>117</sup> Longer  $\text{EG}_n$  SAMs, e.g.,  $n=6$ , prevent protein adsorption, whereas shorter  $\text{EG}_n$  chains do not. The ratio of  $\text{EG}_n$  and unfunctionalized alkanethiol coadsorbates in mixed monolayers can be controlled by varying the relative amounts of  $\text{EG}_n$  and alkanethiol in the solution in which the gold substrate is immersed. Mixed monolayers with a high proportion of  $\text{EG}_n$  prevent protein adsorption. In general, a SAM composed of

$>50\%$   $\text{EG}_n$ , where  $n \geq 3$ , is required to impart resistance to protein adsorption, whereas gold substrates modified with only  $\text{CH}_3$ -terminated SAMs readily adsorb proteins thereby allowing cell adhesion, Fig. 3(B).<sup>117,118</sup> This can be attributed to hydrogen bonding between the  $\text{EG}_n$  units and water, thereby forming a highly hydrophilic monolayer that prevents protein adsorption.<sup>67–69,107,116,119</sup>

Zhu *et al.* used surface plasmon resonance (SPR) to show that gold substrates modified with SAMs of  $\text{EG}_n$  do not completely prevent protein adsorption. These studies showed that proteins are reversibly adsorbed; they are removed upon rinsing the substrate with water.<sup>120</sup> To explore this further, Capadona *et al.* quantified the adsorption of radiolabeled FN on mixed SAMs of methyl-terminated (e.g., alkanethiol) and

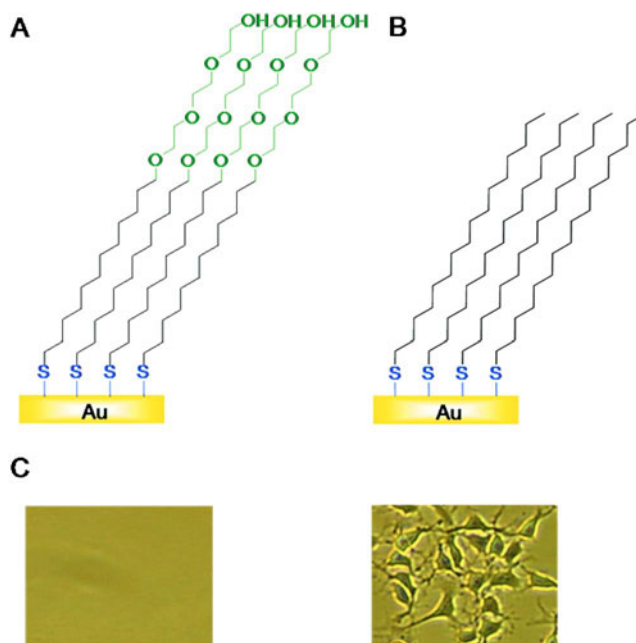


FIG. 3. (Color online) Self-assembled monolayers of alkanethiols on gold: (A) Gold substrate modified with an oligo(ethylene glycol)-terminated alkanethiol,  $\text{EG}_n$ . (B) A simple unfunctionalized (methyl-terminated) alkanethiol monolayer. (C) Substrates modified with  $\text{CH}_3$ -terminated SAMs allow cell adhesion and surfaces modified with mixed  $\text{EG}_3$ - and  $\text{CH}_3$ -terminated SAMs resist cell adhesion.



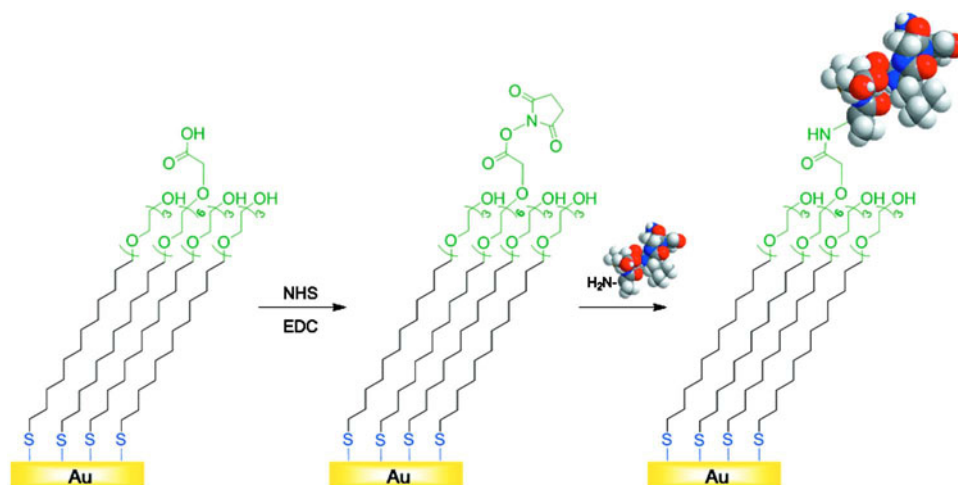


FIG. 4. (Color online) (A) Gold surfaces modified with  $\text{EG}_3$  presenting a terminal hydroxyl group and  $\text{EG}_6\text{-COOH}$ . (B) Activation of the carboxylic acid using EDC/NHS coupling chemistry. (C) Peptide tethered to  $\text{EG}_6\text{-COOH}$ .

$\text{EG}_3$ .<sup>107</sup> Since FN mediates cell adhesion to surfaces, quantifying the amount of FN on the surface and determining conditions under which this can be controlled provides a method to tailor the amount of cell adhesion to the surface. After incubation of mixed-alkanethiol SAMs on gold substrates in radiolabeled FN for 1 h the substrates were incubated in solutions of either phosphate buffered saline (PBS) or 10% newborn calf serum in Dulbecco's modified eagle's medium for 1 or 16 h, and the amount of FN on the surface was quantified. These experiments showed that FN adsorbs to all surfaces modified with SAMs of  $\text{EG}_3$ , albeit at low densities, but that the FN is more easily removed from the substrates modified with a greater proportion of  $\text{EG}_3$ . Adsorbed FN was not eluted from surfaces consisting of only methyl ( $\text{CH}_3$ )-terminated alkanethiols after incubation in PBS for up to 16 h. Similarly, FN adsorbed to substrates presenting a 1:1 mixed monolayer of  $\text{CH}_3$ - and  $\text{EG}_3$ -terminated SAMs could not be removed after incubation in PBS. Adsorbed FN could be eluted in significant quantities from surfaces presenting only  $\text{EG}_3$ -terminated alkanethiols on gold, and after incubation in PBS for 16 h no FN was detected on the surface.

The influence of FN adsorption on cell adhesion to gold substrates modified with mixed monolayers with increasing amounts of  $\text{EG}_3$  was also examined. Gold substrates modified with a mixed monolayer were incubated in FN for 1 h and then challenged with cells. Greater FN adsorption on gold substrates modified with SAMs presenting a smaller amount of  $\text{EG}_3$  correlated with an increase in fibroblast adhesion. Substrates presenting only  $\text{EG}_3$ -terminated SAMs adsorbed FN, but upon subsequent incubation in PBS or 10% serum only background levels of cell adhesion were observed. Surfaces modified with a 1:1 ratio of  $\text{CH}_3$ - and  $\text{EG}_3$ -terminated SAMs on gold substrates that were incubated in media prior to being challenged with cells showed a decrease in cell adhesion after a gentle rinse with PBS. Lastly, gold surfaces consisting of only methyl-terminated adsorbates showed high levels of cell adhesion after incubation in a solution containing FN if they had been rinsed with

PBS or 10% serum prior to cell seeding. These results conclusively show that cell adhesion on SAM-modified substrates is mediated by FN adsorption, and that FN adsorption to substrates can be controlled by modification of the substrates with mixed methyl and oligo(ethylene glycol)-terminated SAMs. This can be attributed to the reversible nature of the FN adsorption to  $\text{EG}_3$ -terminated SAMs, whereas FN irreversibly adsorbs to  $\text{CH}_3$ -terminated SAMs.

Given the effect of SAMs on the adsorption of FN, and consequently on cell adhesion, we set out to modify the oligo(ethylene glycol) terminated monolayers with specific peptide sequences in a controlled manner. It was envisaged that immobilization of a FN fragment from the self-assembly domain onto substrates with a protein adsorption-resistant background would provide opportunities to promote the complex processes involved in the cell-mediated assembly of FN and COL matrices relevant to the development of new biomaterials.<sup>121</sup> Gold substrates were modified with a 19:1 ratio of alkanethiols presenting tri(ethylene glycol) and a hexa(ethylene glycol) bearing a carboxylic acid at the termini,  $\text{EG}_6\text{-COOH}$ , Fig. 4(A). The carboxylic acid group was subject to activation with 1-ethyl-3-(3-dimethylaminopropylcarbodi-imide hydrochloride) (EDC) and *N*-hydroxysuccinimide (NHS), Fig. 4(B), which affords the opportunity to tether peptides via amidation of the *N* terminus or amino side chains, Fig. 4(C). Three peptides were tethered to separate substrates using this EDC/NHS coupling chemistry: (i) the short peptide sequence FN13 (KGGGA-HEEICTTNEGVM), which is from the self-assembly domain of FN (and included a KGGG spacer sequence) and promotes formation of FN fibrils that subsequently mediate cell adhesion,<sup>122</sup> proliferation, and differentiation; (ii) a short RGD-containing peptide sequence, GRGDSPC ("RGD"), which promotes cell adhesion; and (iii) a scrambled FN13 sequence, KGGGTCETNEGEVAMH, to act as a control. This allowed for presentation of specific peptide sequences on a protein adsorption-resistant background.

Cells were seeded on substrates presenting peptide sequences and the assembly of the FN matrix was studied. FN which is assembled into high molecular weight multimers is not soluble in deoxycholate (DOC) detergent, providing us with an assay of the assembly process. Substrates modified with FN13 showed ten times more DOC insoluble FN than controls in which cells were seeded on unmodified substrates, indicating that substrates presenting FN13 enhance FN matrix assembly. Substrates modified with the scrambled FN13 sequence, or RGD alone, showed significantly less assembled FN. Accordingly, presentation of the FN13 fragment on the bioresistive EG<sub>3</sub> monolayer provides a method for the selective deposition and assembly of FN matrices. The density of FN13 presented on surfaces was determined by ellipsometry, and surfaces were modified with varying densities of FN13 to study the effects of peptide density on FN matrix assembly. FN matrix assembly was observed on substrates bearing a density of greater than 8.9 fmol/cm<sup>2</sup> of FN13. Only a minimal basal level of FN assembly was observed at lower densities of the peptide. Thus, substrates can be engineered to present a critical density of FN13 needed to mediate FN matrix assembly, thereby producing materials for biomedical applications that can enhance cell-material interactions and mediate tissue regeneration and assembly.

Although using FN13 is effective in promoting cell-mediated assembly of a robust FN matrix, it is not an adhesive peptide and does not enhance initial cell adhesion. Since initial cell adhesion has been shown to control long-term cell function,<sup>123,124</sup> significant efforts have focused on enhancing cell attachment by the presentation of adhesive ligands on nonfouling supports. Unfortunately, cell adhesion and complex cellular events are often not elicited by the tripeptide RGD, which lacks synergistic binding sites which could be utilized in increasing activity and binding specificity.<sup>58,125,126</sup> Therefore, to increase receptor-ligand specificity and control over cell function, a single 50 kDa recombinant fragment of FN was constructed (FNIII<sub>7-10</sub>) which incorporates both the RGD sequence and its synergistic PHSRN binding site. This ligand mimics the spacing and adhesion characteristics of FN.<sup>116</sup> Gold substrates were modified with SAMs presenting a 98:2 mol ratio of EG<sub>3</sub> and EG<sub>6</sub>-COOH. Substrates were modified using EDC/NHS coupling chemistry with one of three peptides: GRGDSPC (i.e., RGD, an isolated sequence), FNIII<sub>7-10</sub>, and GRGDG<sub>13</sub>PHSRN ("RGD-PHSRN," which mimics the spacing of RGD and PHSRN in FN). The surface density of tethered ligands was quantified using SPR, which showed that substrates tethered the same density of RGD and RGD-PHSRN, whereas a tenfold decrease in tethering density was observed for FNIII<sub>7-10</sub>, which can be attributed to the size of the much larger FNIII<sub>7-10</sub> ligand.

Cell adhesion studies were performed on gold substrates with tethered RGD, RGD-PHSRN, and FNIII<sub>7-10</sub>. After seeding cells on the substrates bearing tethered peptide, the strength of cell adhesion was tested using a centrifugation assay to apply a controlled and reproducible range of forces to cells attached to the substrates. The number of cells that remain adhered to the surface is taken as an indication of the

strength of adhesion.<sup>127,128</sup> Substrates presenting RGD and RGD-PHSRN peptides showed similar cell detachment profiles, indicating similar adhesion strength to the substrates presenting these peptides. However, gold substrates with tethered FNIII<sub>7-10</sub> showed increased amounts of cells on the surface, indicating that they were more tightly adhered. Thus surfaces presenting FNIII<sub>7-10</sub> could be used to enhance cell adhesion in biomaterial applications.

A series of integrin blocking assays was performed to determine which integrins are responsible for cell adhesion onto the peptide modified substrates. The major integrin receptors for FN expressed on the immature osteoblastic cells examined were  $\alpha_5\beta_1$  and  $\alpha_v\beta_3$ . When cells were incubated with an antibody specific for the  $\alpha_5$  subunit, cell adhesion to substrates modified with RGD or RGD-PHSRN was unaffected. However, cell adhesion to substrates modified with FNIII<sub>7-10</sub> was decreased to background levels. Upon incubation in an antibody specific for  $\beta_3$ , cell adhesion to surfaces presenting RGD and RGD-PHSRN showed a 75% decrease, and no appreciable change in cell adhesion was observed on surfaces modified with FNIII<sub>7-10</sub>. These results indicate that cells adhere to substrates presenting RGD and RGD-PHSRN primarily through  $\alpha_v\beta_3$  integrins whereas adhesion of cells to surfaces modified with FNIII<sub>7-10</sub> occurs primarily through  $\alpha_5\beta_1$  integrins, Fig. 5.

To confirm that cell adhesion to FNIII<sub>7-10</sub> modified substrates occurs primarily through  $\alpha_5\beta_1$  integrins and through  $\alpha_v\beta_3$  integrins on surfaces presenting RGD and RGD-PHSRN peptides, cells were seeded and incubated for 4 h on gold surfaces presenting tethered bioligands, and the mode of cell adhesion was determined using integrin staining and by staining for vinculin, a protein found in focal adhesions. Surfaces presenting RGD and RGD-PHSRN showed low levels of focal adhesions containing vinculin and staining for  $\alpha_v\beta_3$  integrins. In contrast, surfaces with tethered FNIII<sub>7-10</sub> showed increased staining for  $\alpha_5\beta_1$  integrin compared to  $\alpha_v\beta_3$  integrins, and greater vinculin staining was observed compared to surfaces modified with RGD or RGD-PHSRN.

Cell adhesion to substrates is also controlled in part by focal adhesions, which are protein complexes that are responsible for cell signaling and cell adhesion to substrates. Studies to control the size and position of focal adhesions were performed in order to determine how they affect the adhesive strength of cells to patterned gold substrates.<sup>115</sup> Gold surfaces were modified using microcontact printing by stamping an unfunctionalized alkanethiol as 2, 5, or 10  $\mu\text{m}$  circular spots. The hydrophobic alkanethiol allows protein adsorption and cell adhesion. The open spaces were then backfilled with EG<sub>3</sub>, which resists adsorption. Cells seeded onto this patterned substrate remained circular and were present only on the methyl-terminated alkanethiol regions. The focal adhesions were visualized using immunofluorescence staining which showed that cells patterned on surfaces within the 10  $\mu\text{m}$  circles had distinct flanges with clustered integrins; no integrins were observed in areas near the center of the pattern. For the smaller circles, cells remained circular and no distinct protrusions were observed, similarly there

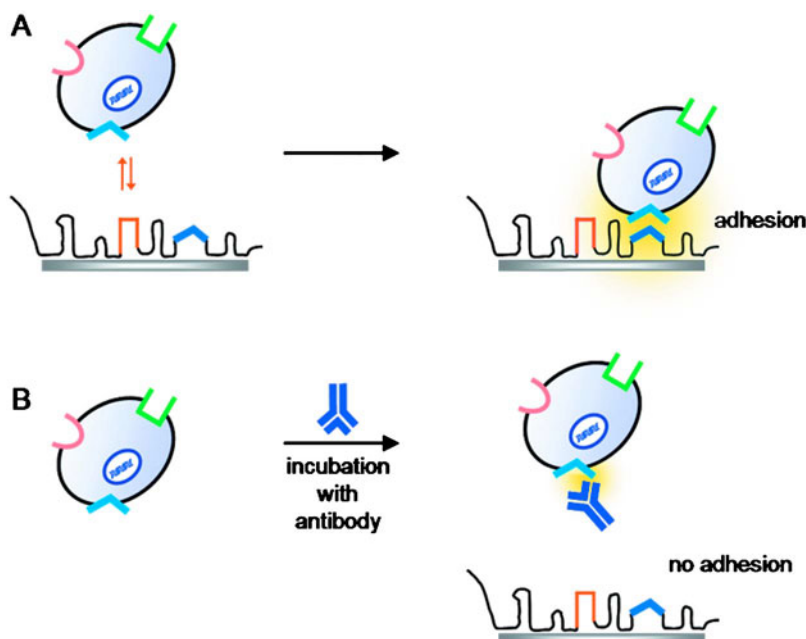


FIG. 5. (Color online) (A) Integrin binding to adhesion sequences presented on substrate surfaces. (B) Antibodies that bind to a specific integrin prevent adhesion of cells through that integrin.

was a uniform distribution of focal adhesions within the cells. However, a larger amount of cytoskeletal proteins, which are needed to maintain the cell shape, were clustered around the edges of the cells.

The adhesive strength of cells to the modified gold substrates was measured using a spinning disk. As the size of the micropattern increases the cell adhesion strength also increases. For example, cells patterned on  $5\ \mu\text{m}$  circles required twice the force to detach them compared to cells patterned on  $2\ \mu\text{m}$  circles. However, the  $10\ \mu\text{m}$  pattern showed only a 20% increase in adhesion strength over the  $5\ \mu\text{m}$  patterned circles, indicating that the increase in adhesion strength may plateau, which can be ascribed to integrin clustering and formation of focal adhesions.<sup>129</sup>

Thus deposition of SAMs provides an excellent model system to tailor substrates in order to resist or direct protein adsorption and cell adhesion. However, gold substrates are poor choices for the development of implantable biomaterials, and SAMs suffer from limited stability.<sup>68,69,71</sup> Accordingly, our attention was drawn to the development of robust hydrophobic polymer brushes on titanium substrates that could be further modified by immobilization of peptides.

### III. POLYMER BRUSHES FOR INTERFACIAL ENGINEERING IN BIOLOGICAL APPLICATIONS

#### A. Introduction

Polymer brushes are assemblies of polymer chains in which one end of the chain is tethered to a surface. The preparation of polymer brushes allows for the design of robust and functional surface coatings.<sup>130</sup> Gold, silver, silicon, glass, and titanium<sup>80,83,84,131–133</sup> substrates have been modified with polymer brushes for an assortment of medical ap-

plications such as diagnostics, cell culture, tissue engineering scaffolds, intraocular lenses, sutures, and orthopaedic applications.<sup>134–136</sup> These polymer brushes provide functional and durable coatings which may be tailored to enhance integration of biomaterials with a host. Table I contrasts the advantages and disadvantages of SAMs and polymer brushes.

The formation of polymer brushes is achieved through either a “grafting to” or a “grafting from” approach. The selective physisorption of one block of a copolymer constitutes a grafting to approach, but given the reversible nature of physisorption it is desirable to use a polymer bearing functional groups which couple to complementary functionality on the substrate to form a covalent bond. Grafting to approaches are limited by steric hindrance; after a few polymer chains attach to the substrate, they impede further attachment by blocking access to remaining binding sites on the surface. This often leads to thin, loosely packed layers of polymer chains, Fig. 6(A).<sup>85</sup>

A “grafting from” approach in which functional groups on the substrate are used to initiate chain growth polymerizations can be used to obtain a higher density of polymer chains on a substrate, Fig. 6(B).<sup>70,80,133</sup> Growth of the polymer chains from the surface relies on the diffusion of small monomers to the propagating chain end, which is less susceptible to steric hindrance than diffusion of a preformed polymer as in the grafting to approaches, Fig. 6(A). Grafting from relies on the introduction of functional groups on the surface to initiate the polymerization of monomers. Polymeric substrates can be functionalized by plasma and glow discharge in the presence of  $\text{O}_2$  or  $\text{N}_2$ . However, this method is not general enough for functionalization of substrates such as metals and inorganics which are of technological impor-

TABLE I. Comparison of the advantages and disadvantages of using SAMs and polymer brushes to control surface properties.

	Self-assembled monolayers	Polymer brushes
Advantages	<p>Simple formation (especially alkanethiols on gold and chlorosilanes on oxides)</p> <p>Molecularly well-defined layers</p> <p>End groups used to tailor surface properties, subject to modification with biological ligands</p>	<p>Long-term stability</p> <p>Options for preparation:</p> <ul style="list-style-type: none"> <li>• grafting from</li> <li>• grafting to</li> </ul> <p>Tunability through choice of monomer or comonomers (e.g., acrylates and styrenes)</p> <p>Variety of polymerization methods SI-ATRP, ROP, NMP, cationic, anionic</p> <p>Greater film thickness; control over brush length</p> <p>Thick film might provide self-healing of defects</p>
Disadvantages	<p>Thin: one molecular layer</p> <p>Limited long-term stability</p> <p>Presence of pinholes and defects</p>	<p>More complex preparation</p> <p>More complex structure</p>

tance, and this method affords little control over the density of functional initiating groups. A more attractive approach is to decorate the solid substrate with a SAM of an initiator-bearing adsorbate. This approach is extremely flexible, with the development of synthetic approaches which make use of cationic,<sup>137,138</sup> anionic,<sup>139</sup> controlled radical, and ring-opening polymerizations (ROPs) (e.g., ring opening of lactide monomers and ring-opening metathesis polymerization of cyclic alkenes).<sup>140–147</sup> Of these methods, controlled radical polymerization of vinyl monomers affords a diverse array of methods to prepare new functional surfaces. These include atom transfer radical polymerizations (ATRP) from alkyl halides,<sup>148–151</sup> nitroxide-mediated polymerizations (NMPs),<sup>151</sup> and reversible addition-fragmentation chain transfer polymerizations from benzyl *N,N*-diethyldithiocarbamates.<sup>152,153</sup>

ATRP is a particularly attractive approach for the preparation of polymer brushes. It is a living polymerization and allows for control of molecular weight and molecular weight distribution and affords the opportunity to prepare block copolymers. Surface-initiated ATRP (SI-ATRP) of methyl methacrylate was first reported from well-defined molecular monolayers formed on glass substrates by Langmuir–Blodgett transfer of an amphiphilic benzyl chloride.<sup>70</sup> Surface-initiated ATRP from an  $\alpha$ -bromo ester terminated trichlorosilane SAM on silicon was reported by Matyjaszewski *et al.*,<sup>154</sup> which was soon followed by polymerization from similarly substituted alkanethiol monolayers formed on gold.<sup>70,85,105</sup> SI-ATRP also provides the opportunity to use a variety of monomers and to tailor the composition and thickness of the brushes by variation of the surface density of initiating end groups, monomer concentration, and polymerization time.<sup>70</sup> The brush density can be controlled by using a mixed SAM consisting of an ATRP initiator and an unfunctional coadsorbate.

The potential to form two-dimensional gradients<sup>155–157</sup> and micron-scale patterns<sup>158–161</sup> of initiator-substituted adsorbates, together with control of the composition of the film by block copolymerization using a variety of polymerization methods<sup>162–164</sup> and monomers, affords a high level of control over the structure and functionality of polymer brushes, which allows for tailoring of the brushes for a wide variety of applications.

## B. Polymer brushes in the design of biomaterials

Hydrophilic oligo(ethylene glycol) substituted polymer brushes have been prepared on gold and silicon substrates to test their ability to resist protein adsorption and cell adhesion. SAMs of adsorbates bearing a bromoisobutyryl group at the termini have been used to initiate ATRP of oligo(ethylene glycol) methacrylate (OEGMA) monomer.<sup>70,85,165</sup> Similar to poly(ethylene glycol) and SAMs of EG<sub>n</sub> on gold, the poly(OEGMA) polymer brushes prevent protein adsorption. Extensive studies of the SI-ATRP of OEGMA polymer brushes on silicon and gold substrates indicate that the length

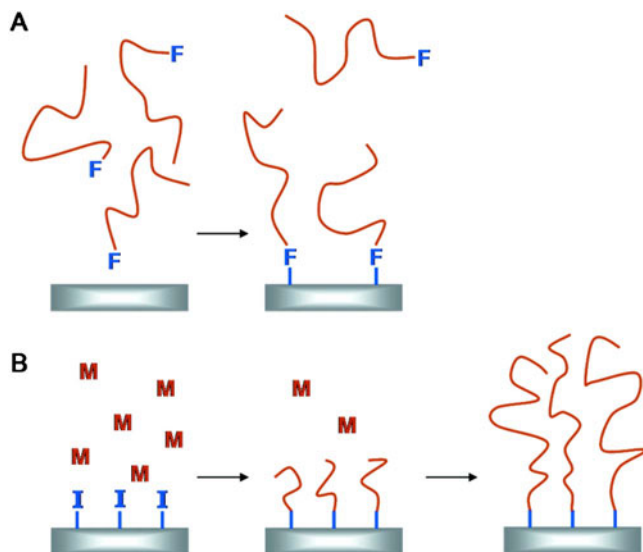


FIG. 6. (Color online) (A) A substrate modified by a grafting to approach in which the initially adsorbed polymer impedes further deposition resulting in a low density of brushes. (B) Modification of a substrate by a grafting from approach in which an initiator-bearing substrate is exposed to monomer to afford thick, dense polymer brushes.



of the polymer brushes increases linearly with time and that the brush film must be at least 100 Å thick in order for optimal prevention of protein adsorption.<sup>70,85</sup> Similarly, polymer brushes formed by surface-initiated nitroxide-mediated polymerization of OEG-substituted styrene imparts bioresistance to silicon substrates.<sup>132</sup>

Messersmith and co-workers used a catechol initiator on titanium substrates to perform SI-ATRP of OEGMA.<sup>83,133</sup> The poly(OEGMA) brushes resisted protein adsorption and cell adhesion for up to 5 weeks. However, the poly(OEGMA) brushes in these studies presented a terminal methyl group. The lack of chemical functionality of the brushes prevented further modification with bioactive peptides that might be used to elicit specific biological responses.

Titanium and its alloys are used extensively for hip and knee joint replacements, as well as in dental and cardiac pacemaker implants. Current titanium implants still suffer from limited integration into the surrounding bone (osseointegration) which ultimately results in loosening and wear, thereby requiring revision surgery.<sup>166,167</sup> Initial attempts to address these limitations focused on physically roughening the titanium surface, but these methods to enhance osseointegration had limited success.<sup>166,168</sup> This led us to explore a possible role for polymer brushes in developing strategies to control the adsorption of proteins and adhesion of cells on titanium substrates. Based on the demonstrated ability to control protein adsorption on model systems consisting of SAMs, we sought to enhance the osseointegration of titanium through decoration of the metal surface with polymer brushes that retain chemical functionality that can be used to immobilize bioligands. Our approach makes use of the SI-ATRP of bioresistive polymer brushes followed by covalent attachment of peptides that signal for cell adhesion and osteoblast differentiation.<sup>169</sup>

### C. Modification of titanium with oligo(ethylene glycol) methacrylate polymer brushes to control cell adhesion

In order to improve on approaches to control cell adhesion by physisorption of peptides onto metallic substrates<sup>133,139,165</sup> we envisaged that SI-ATRP of OEGMA could be used to form a protein adsorption-resistant (i.e., nonfouling) polymer brush system, and that tethering specific peptide sequences to the brushes would allow us to provide signals for cell adhesion and function.<sup>80</sup> Thus, this approach would prevent the nonspecific adsorption of proteins from the biological matrix onto the implant, and enhance selective cell deposition, differentiation, and proliferation. We expect that this approach can be developed so as to promote greater bone growth on the surface, thereby providing a stronger bond between the bone and implant.

Poly(OEGMA) brushes were deposited on a SAM of a dimethylchlorosilane presenting a terminal  $\alpha$ -bromo ester that was used to initiate SI-ATRP of OEGMA to afford poly(OEGMA) brushes, Figs. 7(A) and 7(B). In this case, the OEGMA side chains had a terminal hydroxyl group, which are amenable to modification with 4-nitrophenyl chloroformate

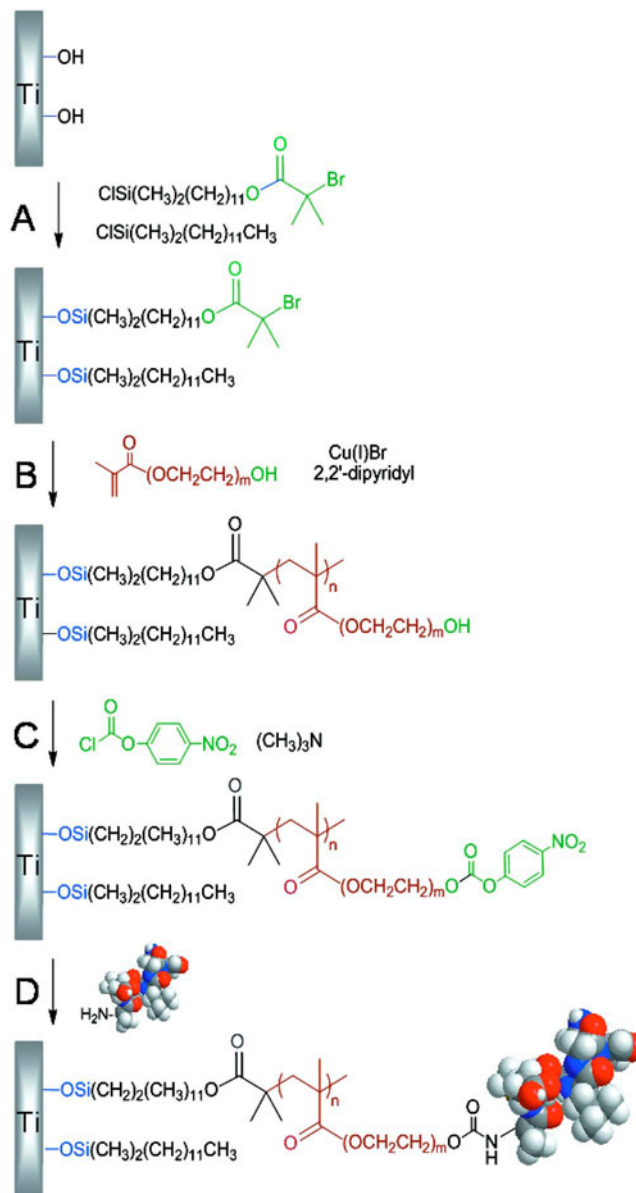


Fig. 7. (Color online) (A) Formation of a mixed SAM by treatment of oxidized titanium with a monochlorosilane bearing a terminal bromoisobutyrate for initiation of SI-ATRP of OEGMA. (B) Poly(OEGMA) brushes with terminal hydroxyl groups. (C) Modification of the hydroxyl groups with NPC. (D) Peptide tethering to the modified hydroxyl end groups of the poly(OEGMA) brushes.

ate (NPC) to produce a 4-nitrophenyl carbonate linkage, Figs. 7(B) and 7(C). The N terminus or an amino side chain of a peptide displaces 4-nitrophenol, thereby forming a new urethane linkage and immobilizing the peptide on the surface, Fig. 7(D).

The surface modification of titanium outlined in Fig. 7 was monitored using specular reflection Fourier-transform infrared spectroscopy (FTIR). The carbonyl absorbance for titanium substrates modified with the bromoisobutyryl initiator ( $1738\text{ cm}^{-1}$ ) shifted to  $1730\text{ cm}^{-1}$  and increased in intensity upon SI-ATRP of OEGMA, corresponding to the deposition on the methacrylate polymer. Modification of the



hydroxyl end groups of the poly(OEGMA) brushes with NPC resulted in the appearance of a second carbonyl absorbance at  $1770\text{ cm}^{-1}$ , which can be attributed to the carbonate linkage. Upon the subsequent treatment with GFOGER, the FTIR spectrum showed a new absorbance at  $1668\text{ cm}^{-1}$  due to the amide linkages. The ability of titanium substrates modified with poly(OEGMA) brushes to resist peptide adsorption and the ability of NPC modified brushes to tether peptide were studied using SPR. The poly(OEGMA) brushes modified with NPC tethered seven times more GFOGER ( $27.8\text{ pmol/cm}^2$ ) through formation of a covalent linkage than unmodified poly(OEGMA) brushes, which can only adsorb peptide by physisorption. In the latter case most of the peptide could be rinsed from the surface, whereas the covalently bound peptide persisted even after rinsing with surfactant solution. The effect of tethered peptide on cell adhesion was explored with fluorescently dyed MC3T3-E1 osteoblast-like cells. Cells rapidly adsorb on unmodified titanium (with an oxide surface) to form a confluent layer, whereas substrates presenting poly(OEGMA) brushes resisted cell adhesion for up to 56 days. Covalent modification of the poly(OEGMA) brushes with GFOGER enhanced cell adhesion. Thus modification of titanium substrates with poly(OEGMA) brushes afforded a nonfouling background to which a specific peptide could be tethered to control cell adhesion.

The ability to successfully tether biomolecules to poly(OEGMA) brushes on titanium substrates was extended to examine the effect of a variety of immobilized peptides on adhesion and enhance osteoblast differentiation. Brushes were modified with different densities of either GRGDSPC (RGD), a linear peptide sequence containing the ubiquitous adhesion sequence, or FNIII<sub>7-10</sub>, a recombinant fragment of FN that presents both the RGD sequence and its PHSRN synergy site. Titanium substrates were modified with poly(OEGMA) brushes, treated with NPC, and incubated in increasing concentrations of peptide to determine the maximum tethering density of the ligands. The maximum peptide tethering densities were determined by SPR as approximately  $6000\text{ fmol/cm}^2$  for RGD and  $1000\text{ fmol/cm}^2$  for FNIII<sub>7-10</sub>. The difference in the density of tethered peptide can be attributed to the relative size of the ligands. An antibody assay that mimics receptor-ligand binding was performed to demonstrate that the ligands are accessible and active on the surfaces. Poly(OEGMA) brushes presenting FNIII<sub>7-10</sub> that were incubated in fluorescently labeled antibody show increasing fluorescence for higher densities of tethered peptide, indicating that the peptide is present in its active form. Unmodified brushes showed only background levels of fluorescence.

#### D. Assays of cell adhesion and function on peptide modified poly(OEGMA) brushes

Integrin binding assays were conducted using titanium substrates modified with poly(OEGMA) brushes with tethered with equimolar densities of RGD or FNIII<sub>7-10</sub>. Cells were incubated in a solution containing antibodies for the  $\alpha_5$

or  $\alpha_v$  integrin, thereby blocking their ability to bind to domains present in surface-immobilized peptides. Cell adhesion on poly(OEGMA) bearing FNIII<sub>7-10</sub> in the presence of  $\alpha_5$ -blocking antibodies was minimal, indicating that cells adhere to immobilized FNIII<sub>7-10</sub> through the  $\alpha_5\beta_1$  integrin receptor. Cells that were incubated in an anti- $\alpha_v$  antibody do adhere to substrates presenting FNIII<sub>7-10</sub>. In contrast, cells incubated with an anti- $\alpha_v$  antibody do not adhere to RGD tethered to brushes, thereby verifying that the  $\alpha_v\beta_3$  integrin is necessary for cell adhesion to substrates presenting RGD.

Focal adhesion kinase (FAK) phosphorylation after 7 days was studied as an indicator of cell signaling. Analysis of the site of FAK phosphorylation in cells on different substrates allowed us to determine the potential influence of the surface modifications on intracellular signaling, specifically for pathways involving integrin signal transduction and osteogenic differentiation.<sup>170,171</sup> Antibodies were used that are specific for three phosphorylated (i.e., activated) tyrosine residues of FAK: (i) Y397, which is important in differentiation and osteogenic pathways; (ii) Y576, which is located in the catalytic portion of the FAK protein, resulting in maximal catalytic activity when it is phosphorylated and thereby priming pathways for osteogenic differentiation; and (iii) Y861, which affects proliferation and differentiation of cells. Experiments were performed on four substrates: unmodified titanium, titanium modified with poly(OEGMA) brushes, and titanium with RGD or FNIII<sub>7-10</sub> tethered to poly(OEGMA) brushes. Increased levels of phosphorylated FAK Y397 and Y576 were observed in cells seeded on surfaces with tethered FNIII<sub>7-10</sub>, corresponding to an increase in indicators for osteoblast differentiation. In contrast, elevated levels of Y861 phosphorylation were observed in cells on unmodified titanium and for surfaces presenting RGD.

To further establish that binding to the modified surfaces controls signaling, cells were incubated with anti- $\alpha_5$  antibody (which prevents cell adhesion to substrates presenting FNIII<sub>7-10</sub>, see above) and seeded onto modified substrates. Substrates modified with FNIII<sub>7-10</sub> showed decreased levels of Y397 and Y576 phosphorylation, whereas no changes in phosphorylation were seen on the substrates modified with RGD or on serum-exposed titanium. These results demonstrate that the  $\alpha_5\beta_1$  integrin selectively binds to FNIII<sub>7-10</sub> and is primarily responsible for FAK phosphorylation of Y397 and Y576.

Cells incubated in a solution of antibody specific for  $\beta_3$  and seeded onto RGD-tethered substrates showed lower levels of FAK phosphorylation. This indicates that binding of the  $\beta_3$  integrin to RGD is responsible for regulation of phosphorylation of Y861. Thus, these results show that cells incubated on poly(OEGMA) brushes with tethered FNIII<sub>7-10</sub> show differential cell signaling and, moreover, increased levels of osteoblast-related signaling markers compared to those on unmodified titanium substrates or on poly(OEGMA) brushes with tethered RGD. Thus, the surface-immobilized adhesion sequences not only increase cell adhesion but also exert control over more downstream cellular function.

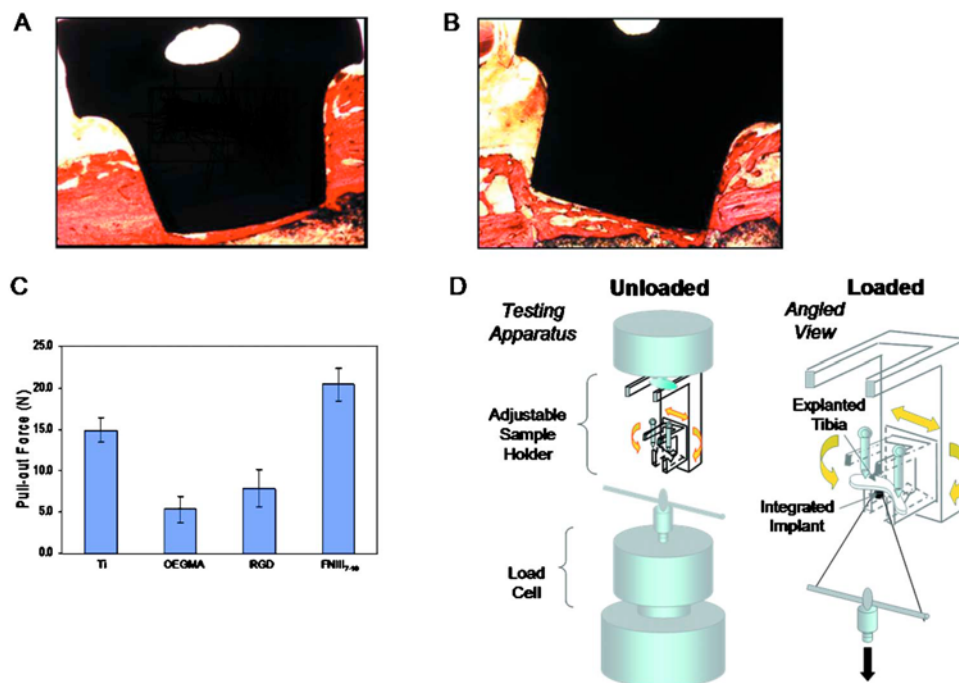


FIG. 8. (Color online) (A) Cross section view of titanium implant with peptide tethered to poly(OEGMA) brushes in rat tibia. (B) Cross section view of titanium implant modified with poly(OEGMA) brushes. (C) Pull-out forces required to removed implants with various surface treatments from rat tibiae. From Ref. 177. Copyright © 2008 by from Elsevier. Reprinted and modified in part by permission of Elsevier. (D) Schematic of apparatus used to determine the pull-out force.

### E. Osteoblastic differentiation assays on peptide modified poly(OEGMA) brushes

Assays were conducted to determine if the bioligands tethered to the poly(OEGMA) brushes stimulated expression of markers that are associated with osteoblast differentiation. Unmodified titanium, titanium substrates modified with poly(OEGMA) brushes, and poly(OEGMA) brushes with tethered RGD or FNIII<sub>7-10</sub> were seeded with rat bone marrow stromal cells for 7 days. Gene expression was assessed using reverse transcription polymerase chain reaction for the Runx2/Cbfa1 transcription factor, which is necessary for bone formation,<sup>172</sup> and the late osteoblastic markers osteocalcin (OCN) and bone sialoprotein (BSP). Surfaces with tethered FNIII<sub>7-10</sub> showed elevated levels of Runx2/Cbfa1, OCN, and BSP compared to the other surfaces, indicating enhanced osteoblast differentiation on these substrates. In addition, alkaline phosphatase activity was increased on surfaces functionalized with FNIII<sub>7-10</sub>, which also is indicative of enhanced osteoblast differentiation.

Matrix mineralization was determined using an end-point functional marker, calcium incorporation. This analysis showed two times more mineralization on titanium substrates with tethered FNIII<sub>7-10</sub> to poly(OEGMA) brushes compared to titanium substrates with RGD tethered to poly(OEGMA) brushes.

Taken together these results show enhanced osteoblast differentiation in primary bone marrow stromal cells cultured on titanium substrates modified FNIII<sub>7-10</sub> tethered to poly(OEGMA) brushes. This further illustrates the ability to use

our molecularly engineered surface architectures to direct cell function in a manner relevant to the design enhanced bone implants.

### F. In vivo osseointegration of peptide modified poly(OEGMA) brushes

The increase in osteoblast differentiation on substrates modified with FNIII<sub>7-10</sub> tethered to poly(OEGMA) brushes observed *in vitro* was promising for the development of new bioactive materials. Accordingly, *in vivo* studies were conducted to examine the effectiveness of these surfaces in rigorous animal models. Custom-made clinical grade titanium cylinders were modified with either unmodified poly(OEGMA) brushes or RGD or FNIII<sub>7-10</sub>-tethered brushes. The cylinders were press fitted into 2.0 mm diameter holes in the proximal tibial metaphyses of mature Sprague-Dawley male rats. After 4 weeks of implantation the tibiae were harvested. Osseointegration was analyzed by histomorphometry, and implant mechanical fixation was determined by measurement of pull-out force.

Histomorphometric analyses were performed to determine the contact area between the implant and bone. Titanium cylinders with FNIII<sub>7-10</sub> tethered to poly(OEGMA) brushes had more bone tissue around the implant compared to all other surfaces, with a 70% increase in bone contact area compared to unmodified titanium, the current clinical standard orthopedic implant material, Fig. 8(A). The mechanical fixation of the implants was tested using a pull-out test, in which the force required to remove the implant was deter-

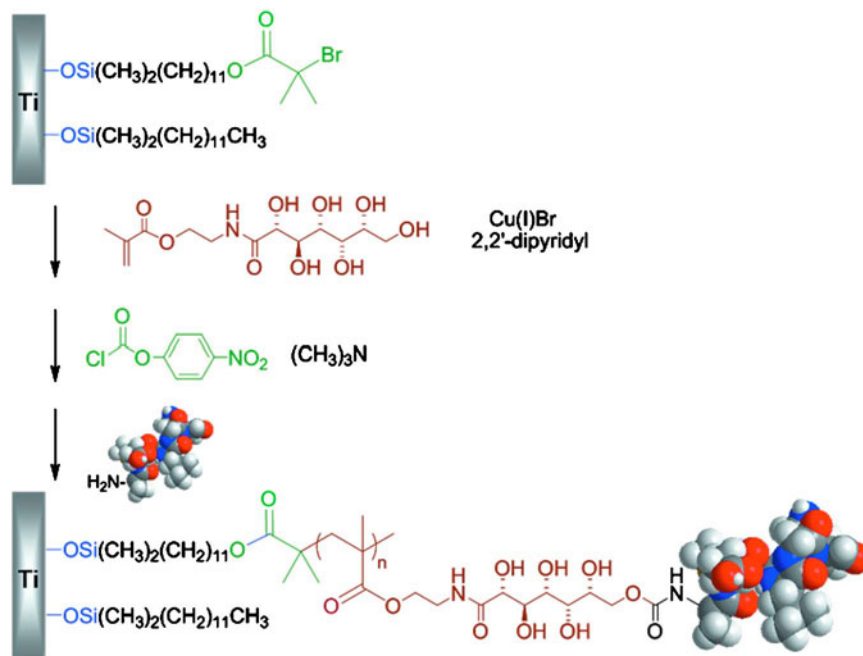


FIG. 9. (Color online) Synthesis of poly(GAMA) brushes, modification of hydroxyl end groups with NPC, and peptide tethering.

mined, Fig. 8(B). Again, titanium cylinders modified with poly(OEGMA) brushes presenting FNIII<sub>7-10</sub> outperformed unmodified titanium. Notably, removal of the implants modified with FNIII<sub>7-10</sub> from bone required a greater force than unmodified titanium, Fig. 8(C). The poly(OEGMA) brushes and RGD-tethered brushes required lower pull-out forces, consistent with the results of *in vitro* bioassays.

Thus, *in vitro* studies of titanium substrates modified with ligands tethered to poly(OEGMA) brushes selectively enhance cell adhesion and osteoblast differentiation of rat bone

marrow stromal cells. Polymer brushes presenting FNIII<sub>7-10</sub> provides greater binding specificity and shows increased osteoblast differentiation than substrates presenting a short immobilized RGD sequence. *In vivo* studies using titanium cylinders modified with poly(OEGMA) brushes presenting FNIII<sub>7-10</sub> show enhanced osseointegration compared to unmodified titanium poly(OEGMA) brushes bearing RGD. These encouraging results motivated continued exploration of our strategy to present selected peptides on bioadhesion-resistant hydrophilic brushes for the development of im-

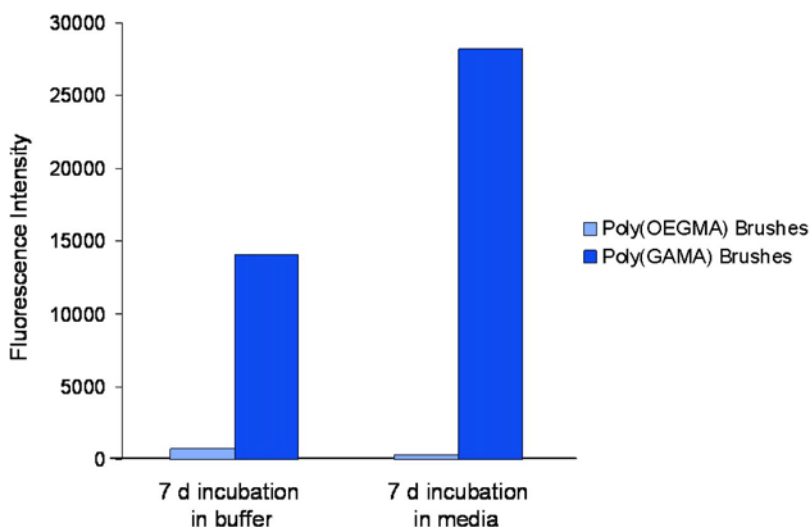


FIG. 10. (Color online) Enzyme-linked immunosorbent assay of poly(OEGMA) brushes after long-term incubation in buffer or media showed little peptide adsorption. Poly(GAMA) brushes showed an increase in peptide adsorption upon 1 week incubation in buffer, and upon incubation in media poly(GAMA) brushes showed a significant increase in peptide adsorption compared to poly(OEGMA) brushes.

proved orthopedic implant materials (Sec. IV).

#### IV. CONCLUSIONS AND PERSPECTIVES

The use of self-assembled monolayers and polymer brushes affords strategies to provide control over protein adsorption and cell adhesion on biomaterials. Deposition of SAMs of EG<sub>3</sub> on gold and poly(OEGMA) brushes on titanium imparts resistance to nonspecific adsorption of proteins. Covalent immobilization of adhesion peptides to the bioresistive surfaces presents opportunities to elicit specific cell adhesion and responses. While SAMs of alkanethiols on gold are well suited as models for analyses of the effects of surface modifications on bioadhesion, we have advanced our study of peptide modified poly(OEGMA) brushes on titanium to *in vivo* experiments. Poly(OEGMA) brushes presenting the FNIII7-10 fragment bind cells primarily via the  $\alpha_5\beta_1$  integrin and promote osteoblast differentiation. Enhanced osseointegration of titanium implants with rat tibiae was demonstrated in a rigorous animal model. Greater density of new tissue growth around the modified substrate results in a stronger mechanical union between the implant and bone. Thus, the molecular-scale interfacial engineering of biomaterials presents an attractive pathway toward the design of enhanced orthopedic implants.

In addition to their use in medical implants, the use of polymer brushes might be extended to other bioapplications such as biosensors. This approach could be used to overcome problems of biofouling of optical and electronic sensing surfaces by nonspecific adhesion of proteins and cells. This adsorption may be mitigated by providing a nonfouling background bearing selected proteins, peptides, or antigens to bind to specific components present in a complex mixture. To achieve this goal, poly(OEGMA) brushes that resist nonspecific protein adsorption could be used to coat sensor chips and, as we have demonstrated, the hydroxyl end groups of the brushes could then be modified to tether specific bioligands.<sup>173</sup> For example, in a demonstration of this principle, immunoglobulin (IgG) has been immobilized on the NHS-bearing poly(OEGMA) brushes on silicon substrates.<sup>174</sup> This was explored as a prototype for a sensor based on binding of fluorescently labeled anti-IgG antibody in a micropatterned array.<sup>175</sup>

Given the successful demonstration that poly(OEGMA) brushes resist *in vitro* and *in vivo* adsorption of proteins and that immobilization of specific adhesion sequences can be used to promote cell adhesion and functions, we have recently extended this approach to explore the surface modification of titanium with saccharide-based polymer brushes. SI-ATRP of the 2-gluconamidoethyl methacrylate (GAMA) monomer on titanium substrates<sup>176</sup> affords poly(GAMA) brushes which are subject to modification with peptides, Fig. 9. The brushes exhibit increased resistance to protein and cell adhesion in short-term experiments (i.e., 1 h incubation of substrates in media with cells). However, resistance was lost in our initial long-term cell adhesion studies in which poly(GAMA) brushes were incubated in serum-containing media for 1 week and then exposed to cells. The loss of resis-

tance to adhesion from incubation in serum-containing media might be attributed to adsorption of proteins or to degradation of the brushes. To examine these mechanisms, titanium substrates modified with poly(GAMA) brushes and poly(OEGMA) brushes were incubated in either serum-containing media or serum-free buffer which contained a biotin-substituted GFOGER peptide. An enzyme-linked immunosorbent assay (ELISA) was performed with an antibody specific for biotin. Substrates modified with either type of polymer brush, incubated for 1 h in either media or buffer containing biotinylated GFOGER, showed only low levels of adsorbed peptide. However, after a 1 week incubation, poly(OEGMA) brushes still showed only low levels of GFOGER adsorption and the poly(GAMA) brushes incubated in buffer showed a higher level of adsorption a much greater amount of peptide adsorbed to poly(GAMA) brushes on titanium-modified substrates incubated in media, Fig. 10. Assuming that the increase in peptide adsorption could be correlated with an increase in protein adsorption over time, this explains the enhanced cell adhesion observed in long-term studies.

The approaches outlined in this review provide strong motivation to further extend the use of molecularly designed interfaces for biological applications and to assess the long-term *in vivo* performance for these engineered coatings. These approaches lend themselves to application in a number of technologies including sensing, enhanced tissue repair, and other regenerative medicine. We anticipate that such molecular approaches to interfacial engineering will provide broadly applicable platforms for the reduction of nonspecific biological reactions and the controlled presentation of bioadhesive motifs to elicit directed cellular responses.

#### ACKNOWLEDGMENTS

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